EFFECTS OF PANTOPRAZOLE ON XENOBIOTIC METABOLIZING ENZYMES IN RAT LIVER MICROSOMES: A COMPARISON WITH OTHER PROTON PUMP INHIBITORS

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ABSTRACT:

The effects of pantoprazole on xenobiotic metabolizing enzymes in rat liver microsomes were examined. Groups of female Sprague-Dawley rats were orally administered pantoprazole and other proton pump inhibitors, omeprazole and lansoprazole, at 5, 50, or 300 mg/kg/day for 7 days, followed by assays to detect changes in the levels of liver microsomal protein, cytochrome P450, cytochrome b5, NADPH cytochrome c reductase, and drug metabolizing enzyme activities. Increases in total cytochrome P450 contents were evident after a 7-day high-dose administration of all the proton pump inhibitors tested, and the increase by treatment with pantoprazole was less than that with lansoprazole. The three proton pump inhibitors increased the enzymatic activities and cytochrome P450 enzyme levels of CYP1A, CYP2B, and CYP3A. CYP1A was less induced with pantoprazole than with omeprazole or lansoprazole. In contrast, CYP2B was more strongly induced with pantoprazole than with other proton pump inhibitors. NADPH cytochrome c reductase was induced with omeprazole and pantoprazole. The present results suggest that enzyme induction differs among these proton pump inhibitors not only quantitatively but also qualitatively.

PAN1, sodium 5-(difluoromethoxy)-2-[[3,4-dimethoxy-2-pyridinyl)methyl] sulfonyl]-1H-benzimidazolide sesquihydrate, is a drug used in the treatment of peptic ulcers (fig. 1). It is a PPI which binds H+/K+-ATPase via a disulfide bond, thereby blocking the active transport of H+ into the gastric lumen (1). Metabolic biotransformations of PAN by P450s have been investigated in detail (2). Phase I metabolic reactions of PAN include mainly S- and methyl group oxidations and aromatic hydroxylations, all of which are typical reactions catalyzed by P450. The hydroxylated metabolites then undergo phase II metabolism, i.e. glucuronidation or sulfation, and are then excreted chiefly in urine. PAN is more highly water soluble and well absorbed from the alimentary tract than OM and LAN which are also substituted benzimidazole PPIs (3, 4).

To date there are no reported signs of toxicity in any animal species for PAN, and its oral LD50 values for mice and rats are reportedly greater than 1 g/kg. It has exhibited no clinically significant interactions with theophylline, antipyrine, diclofenac, digoxin, nifedipine, diazepam, warfarin, and phenytoin (5–9). However, OM increases plasma levels of concomitantly administered diazepam, phenytoin, and certain other drugs, and this effect is attributed to inhibition of P450 (10). In contrast, the repeated administration of OM and LAN also results in the induction of CYP1A2 in vivo in man (11–13), and in vitro studies using cultured human hepatocytes suggest strong induction of CYP1A and CYP3A subfamilies by OM and LAN (14, 15). Thus, PPIs are not only substrates for P450 but also undergo integrated interactions with P450, resulting in either inhibition or

1 Abbreviations used are: PAN, pantoprazole; OM, omeprazole; LAN, lansoprazole; MC, 3-methylcholanthrene; PB, phenobarbital; U, untreated; CMC, carboxymethyl cellulose; EROD, ethoxyresorufin O-deethylation; PROD, pentoxyresorufin O-depentylation; POD, phenacetin O-deethylation; T6H, testosterone 6β-hydroxylation; P450, cytochrome P450; b5, cytochrome b5.

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induction. Some reports are discussed about the induction of CYP1A and CYP3A by OM and LAN (14, 15); however, little information is available in the literature concerning the induction of xenobiotic metabolizing enzymes by PAN. The present study examines the effects of daily oral administration of PAN on xenobiotic metabolizing enzymes in female rats in comparison with OM and LAN by quantifying enzyme levels and measuring specific P450 activities.

Materials and Methods

Chemicals. PAN was supplied by Byk Gulden Lomberg Chemische Fabrik GmbH (Konstanz, Germany). OM and LAN were synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). The chemical structures of these compounds are shown in fig. 1. MC, aminopyrine, 7-ethoxy coumarin, formaldehyde, umbelliferone, phenacetin, 4-acetamidophenol, ethoxyresorufin, pentoxyresorufin and resorufin were purchased from Sigma Chemical Co. (St. Louis, MO). N-Butyl-p-aminophenol was obtained from ICN Biochemicals, Inc. (Lisle, IL), and PB and MgCl₂ from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [14C]-Testosterone and unlabeled testosterone were purchased from DuPont/NEN, Inc. (Boston, MA) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively. NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other reagents used were of analytical grade.

Animals and Treatment. Twelve groups of SD female rats (age: 7 weeks, body weight: 150–180 g) were used and assigned to treatment groups consisting of 0.5% CMC solution, MC, PB, OM (5, 50, or 300 mg/kg/day), OM (5, 50 or 300 mg/kg/day), or LAN (5, 50, or 300 mg/kg/day). The MC-treated group received an MC suspension in corn oil at 30 mg/kg ip once for 1 day, and the PB-treated group received a solution of PB in physiological saline at 80 mg/kg ip once for 1 day. PAN, OM, and LAN, each suspended in 0.5% CMC solution, were administered orally once a day for 7 days. All animals were weighed at each dose administration and immediately before sacrifice. Rats were sacrificed 24 hr after the last dose. Liver microsomes were prepared using standard ultracentrifugation techniques (16).

Determination of Protein, P450, b₁, Content, and NADPH Cytochrome c Reductase Activity. Determination of total liver microsomal protein was performed using the method of Lowry et al. (17) with bovine serum albumin (fraction V, Sigma Chemical Co.) as a standard. Determination of P450 and b₁ levels were performed as described by Omura and Sato (16, 18). NADPH-cytochrome c reductase activity was measured by the method of Phillips and Langdon (19), with one unit defined as the activity required to reduce 1 µmole of cytochrome c in one min at 25°C.

Assay of Drug Metabolizing Enzyme Activities. Aminopyrine N-demethylation assay was performed by measuring the amounts of formaldehyde, as described in Mazel (20). Ethoxy coumarin O-deethylation activity was determined by measuring umbelliferone formed according to the fluorometric method of Greenlee and Poland (21). EROD and PROD activities were measured by the methods of Polak and Feults (22) and Lubet et al. (23). The fluorometric determination of the increase in resorufin was performed using a Hitachi Model 650–60 spectrophotometer. POD activity was determined by a slight modification of the HPLC method of Kokward et al. (24), by measuring 4-acetamidophenol resulting from deethylation of phenacetin. The HPLC system (Shimadzu Co., Kyoto, Japan) included the following: SCL-10A, system controller; SPD-10A, UV detector; LC-10AD, pump; and DGO-4A, degasser. The column used was Inertsil ODS (GL Science, Tokyo, Japan). The wavelength detector was set to 243 nm and the flow rate was maintained at 1.0 ml/min; using MeOH/H₂O as the mobile phase controlled by a gradient program (0–8 min, 30/70; 8–13 min, 55/45; 13–18 min, 30/70). TGRA activity was determined by a slight modification of the method described by Wexman (25). The radioactivity in each assay was 75.75 nCi. Each sample was applied onto a silica gel thin layer chromatographic plate (SiZ25OF-PA, 0.25 mm thickness, J. T. Baker, Inc., Phillipsburg, NJ) and developed with a solvent mixture composed of chloroform/ethyl acetate/ethanol (4/1/0.7, v/v/v). The RI value of the 6b-hydroxy metabolite was 0.43. The plate was analyzed for radioactivity using a bioimaging analyzer system (BAS 2000, Fuji Photo Film Co., Ltd., Tokyo, Japan). The substrate concentrations of aminopyrine and ethoxy coumarin, ethoxyresorufin, pentoxyresorufin, phenacetin and testosterone were 5 mM, 0.2 mM, 10 μM, 10 μM, 0.5 mM, and 25 μM, respectively.

Determination of P450 Isozyme Contents by Western Blot Analysis. Liver microsomal P450s from treated and control rats were quantified by Western blot analysis. The primary and secondary antibodies were diluted by 0.1 M phosphate buffer (pH 7.4) and 10% of Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Microsomal protein (0.5 μg) was loaded on the each well. The specific antibodies used included anti-CYP1A1, anti-CYP2B1, anti-CYP2C6, anti-CYP2E1, anti-CYP3A2, anti-CYP4A1, and anti-NADPH cytochrome c reductase, which were all purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). The immunoblotting procedure was performed using the methods of Laemmli (26) and Guengerich et al. (27). Individual P450 content was measured with an image analyzer (BIO-RAD, M & S Instruments, Tokyo, Japan). All the samples tested were within the calibration range, and the calibration curve exhibited a linearity of r² = 0.76–0.97.

Data Presentation and Statistical Analysis. All values are presented as mean ± SD. Groups were compared by ANOVA, followed by Fisher’s PLSD method for multiple comparisons. A p value < 0.05 was considered statistically significant.

Results

Changes in Liver Weight and Liver Microsomal Protein Content in Rats after One Week of Treatment. Liver weight and liver microsomal protein content were determined from the rat repeated dose of PAN, OM, LAN, PB, and MC. Treatment with PAN, OM, or LAN did not change liver weight, and increased liver weights were observed for the animals treated with PB and MC when compared with weights of the control animals (data not shown). No significant increase in hepatic microsomal protein levels was observed in any group.

Effects of PAN Treatment on Microsomal P450 and b₁ Contents and on NADPH Cytochrome c Reductase Activity. A significant increase in liver microsomal P450 was observed at a high dose of PAN (table 1). High doses of OM and LAN also produced significant increases in the P450 contents, and the induction by LAN was significantly greater than that by PAN. On the other hand, a middle or low dose of any PPI tested did not increase the P450 contents (table 1). Microsomal b₁ contents increased significantly after treatment with PAN, OM, and LAN at the high doses, and the contents in the OM- and LAN-treated groups were significantly higher than the PAN-treated group (table 1). The NADPH cytochrome c reductase activity also increased significantly in animals receiving all of the 3 PPIs at the high doses (table 1).

Effects of PAN Treatment on Drug-Metabolizing Enzyme Activities. OM and LAN at the high doses significantly increased ethoxy coumarin O-deethylation activities (ca. 2.6 and 2.8 times control, respectively), but PAN did not (ca. 1.6 times control). On the other hand, PAN at the high dose level was the only compound tested that significantly elevated aminopyrine N-demethylation activity (ca. 1.3 times control). The increase in this activity with PAN corresponded to 60% of levels induced by PB treatment (ca. 2.1 times control).

EROD activity was slightly increased over control in the PAN high-dose treated group, but not to a significant extent. In contrast, significant increases over control levels were noted for the OM high-dose and LAN middle- and high-dose treated groups, i.e. 4.8-, 4.8-, and 5.5-fold, respectively, all of which were significantly higher than PAN-treated groups at the corresponding doses. In the positive control MC-treated group, a 9.5-fold increase in activity over the control. MC-treated group, a 9.5-fold increase in activity over the
Determined (Table 2). The PAN-treated group exhibited an increased OM, or LAN at the highest dose, 300 mg/kg/day for 7 days were.

Specific P450 levels in liver from rats orally administered PAN, sis.

2.2-fold increases in this activity, respectively (fig. 5).

High doses of OM and LAN also significantly increased this activity, compared with that of the control group. There was a 24-fold increase in activity with the positive control PB-treated group (fig. 3).

Intergroup comparisons of POD activity demonstrated 3.0-, 3.1-, and 2.5-fold significant increases in the PAN, OM, and LAN at the high-dose treated groups, respectively, as compared with control activity (fig. 4). Similar to EROD activity, the LAN middle-dose group as well as the high-dose group displayed a marked increase in POD activity as compared with control (2.7-fold).

Treatment with high dose of PAN resulted in a significant, 2.5-fold, increase in T6H activity, compared with that of the control group. High doses of OM and LAN also significantly increased this activity (2.9- and 1.8-fold, respectively). PB and MC resulted in 7.3- and 2.5-fold significant increases in the PAN, OM, and LAN at the high-dose treated groups, respectively, as compared with control.

Measurement of P450 Enzyme Levels by Western Blot Analysis. Specific P450 levels in liver from rats orally administered PAN, OM, or LAN at the highest dose, 300 mg/kg/day for 7 days were determined (table 2). The PAN-treated group exhibited an increased CYP1A1 content (6.1-fold greater than control). This 6-fold increase, however, was significantly less than that found in the OM- and LAN-treated group (8.2- and 9.3-fold control, respectively). There was a 18.8-fold increase in the content with MC-treated group. CYP1A2 content also increased significantly after treatment with PAN, OM, LAN, and MC (3.6-, 3.9-, 6.3- and 9.9-times the control level, respectively), and the increase in the content in the PAN-treated group was significantly lower than that in the LAN-treated group. A 2.9-fold increase in CYP2B1 occurred in the PAN-treated group, whereas OM and LAN resulted in 2.0- and 2.7-fold increases in this activity in the PAN, PB treatment resulted in a 14.9-fold increase. The increase in CYP2B1 by the treatment of PAN was significantly greater than that found by that of OM. Animals treated with PAN exhibited a 5.0-fold increase in CYP2B2 content, which was significantly greater than that seen in the OM- and LAN-treated groups. Microsomal CYP3A2 content increased 1.9-, 2.9-, and 3.0-fold in the PAN-, OM- and LAN-treated groups, respectively, but not to a significant extent.

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg/day)</th>
<th>Cytochrome P450 (nmol/mg protein)</th>
<th>Cytochrome b5 (nmol/mg protein)</th>
<th>NADPH-cytochrome c Reductase (U/mg protein)</th>
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<tr>
<td>Treated</td>
<td></td>
<td></td>
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<tr>
<td>CMC 0.50%</td>
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<td>0.643 ± 0.098</td>
<td>0.456 ± 0.014</td>
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<td>PAN 5</td>
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<td>0.641 ± 0.059</td>
<td>0.431 ± 0.013</td>
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<td>PAN 50</td>
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<td>0.700 ± 0.022</td>
<td>0.481 ± 0.032</td>
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<td>PAN 300</td>
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<td>0.822 ± 0.003a</td>
<td>0.605 ± 0.019a</td>
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<td>0.660 ± 0.054</td>
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<td>OM 50</td>
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<td>LAN 5</td>
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<td>0.603 ± 0.059</td>
<td>0.376 ± 0.034b</td>
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<td>LAN 50</td>
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<td>0.717 ± 0.119</td>
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<td>LAN 300</td>
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<td>MC 30</td>
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<td>1.11 ± 0.135b</td>
<td>0.653 ± 0.055b</td>
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<td>PB 80</td>
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<td>1.68 ± 0.168ab</td>
<td>0.707 ± 0.066b</td>
<td>0.194 ± 0.017ab</td>
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</table>

Each value represents the mean ± SD for 3 rats. Groups were compared by ANOVA, followed by Fisher’s PLSD method for multiple comparisons.

† Significant difference from PAN-treated group at the respective dose, * Significant difference from control group.

### Fig. 2

**EROD activity in rat liver microsomes after repeated daily administration of PAN, OM, or LAN.**

Groups were compared by ANOVA, followed by Fisher’s PLSD method for multiple comparisons. * Significant difference from control group, † Significant difference from PAN-treated group at the respective dose, p < 0.05.

### Fig. 3

**PROD activity in rat liver microsomes after repeated daily administration of PAN, OM, or LAN.**

Groups were compared by ANOVA, followed by Fisher’s PLSD method for multiple comparisons. * Significant difference from control group, † Significant difference from PAN-treated group at the respective dose, p < 0.05.
In contrast, a 10.1-fold significant increase was observed in the rats given PB. No significant change was observed in CYP2C6, CYP2E1, or CYP4A1, except for induction of CYP2C6 and CYP4A1 by LAN and PB. NADPH cytochrome c reductase content increased significantly in the PAN, OM, and PB-treated groups (2.3-, 1.8-, and 3.1-fold, respectively) and remained unaltered after treatment with MC or LAN.

**Discussion**

To determine the ability of drugs to induce xenobiotic metabolizing enzymes, assays for P450 and related enzyme levels in control and drug-treated animals are usually performed. In the present induction experiments, increases of total hepatic P450 protein content were in the order PAN = OM < LAN at the dose of 300 mg/kg/day. Although the effect of PAN was lower than that of OM, the values for the two compounds were not statistically different. As shown in this study, the PPI-treated groups did display marked changes in the profiles of P450 isozyme contents compared with those seen for the control group. We successfully determined the ability of PAN and other PPIs to induce drug metabolizing enzymes in this study by measuring enzyme activities specific to respective P450s and by quantifying the enzyme protein levels themselves with Western blot analysis.

Since EROD activity in the rat is a reaction specifically involving CYP1A1 (28, 29), assays of this activity are very useful for detecting induction of this isozyme. Here, an increase in liver microsomal EROD activity occurred in rats treated with high doses of the PPIs, the increase being greatest with LAN (5.5-fold control) and least with PAN (2.2-fold). These activity levels are consistent with the quantitative data obtained by Western blot analysis: The CYP1A1 level was highest with LAN and lowest with PAN. In addition, both CYP1A1

<table>
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<tr>
<th>Anti-P450</th>
<th>P450 Contents (pmol P450/mg protein)</th>
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<tr>
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<tr>
<td>CYP1A1</td>
<td>42.7 ± 30.1</td>
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<tr>
<td>CYP1A2</td>
<td>38.0 ± 10.0</td>
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<tr>
<td>CYP2B1</td>
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<td>CYP3A2</td>
<td>50.1 ± 20.8</td>
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<tr>
<td>CYP4A1</td>
<td>12.7 ± 1.2</td>
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<tr>
<td>NADPH cyt c</td>
<td>15.7 ± 8.2</td>
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<tr>
<td>Total P450</td>
<td>0.64 ± 0.998</td>
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</table>

Female SD rats (7 weeks of age) were administered PAN (300 mg/kg/day), OM (300 mg/kg/day), and LAN (300 mg/kg/day) orally for 7 days. MC (30 mg/kg/day) and PB (80 mg/kg/day) intraperitoneally for 3 days and 7 days, respectively. All were killed 24 hours after the last dose. Each value represents the mean ± SD for 3 rats.

CYP, cytochrome P450; NADPH cyt c, NADPH cytochrome c reductase.

Groups were compared by ANOVA, followed by Fisher’s PLSD method for multiple comparisons.

a Significant difference from control group, p < 0.05.

b Significant difference from PAN-treated group, p < 0.05.

c Significant difference from control group, p < 0.05.

† nmol P450/mg protein. Total cytochrome P450 was measured by the reduced-CO spectral method.
and CYP1A2 were induced with LAN at a lower dose (50 mg/kg/day) where no induction with PAN or OM was observed.

Burke and Mayer (30) demonstrated that in hepatic microsomes prepared from rats treated with PB, PROD activity is induced 100-fold greater than the dealkylation of other n-alkyl substituted resorufin, suggesting that this reaction is specifically mediated by CYP2B1 (28, 31, 32). The results here of PROD activity and Western blot analysis show an increase in the CYP2B2 subfamily following treatment with three PPIs, especially with PAN. Unlike the induction of CYP1A, the order of induction of this enzyme was PAN > OM, LAN. Upon considering the P450 induction by PPIs, only induction of CYP1A and CYP3A have been investigated in detail, i.e. OM and LAN induce CYP1A and CYP3A in humans (11–15); however, there is no information about induction of CYP2B. This is the first report to evaluate the induction of CYP2B with PPIs. These 3 PPIs are mixed type inducers of CYP1A, CYP2B, and CYP3A. PAN induced CYP2B preferentially rather than CYP1A. In contrast, OM and LAN induced CYP1A rather than CYP2B.

Phenacetin is metabolized chiefly by CYP1A2 (33), whereas POD activity was shown to be increased not only by MC but also by PB treatment at high substrate concentrations (33–35), probably because CYP2B also made contribution to the activity at high substrate concentrations. Here MC and PB treatment resulted in comparable activities when the assays were carried out at the high substrate concentration, thereby supporting previous findings. POD activity increased 2.5- to 3-fold after treatment at the highest doses tested with all of the PPIs and the degree of increase did not appreciably differ among the compounds, while LAN increased it greater than PAN at the middle dose. CYP3A was induced by OM and LAN in human hepatocytes as reported previously (14), and in this study it was induced by all 3 PPIs, also in rats.

It is known that NADPH cytochrome c reductase is induced by PB but is not affected by MC (36, 37). The response of microsomal NADPH cytochrome c reductase contents to treatment with the 3 PPIs tested here varied greatly depending upon the treatment, i.e. treatment with PAN resulted in increase in the contents, similar to those seen with PB treatment, whereas LAN treatment had no effect, similar to the MC-treated rats.

It is not established whether PPIs result in induction of the enzymes at clinical levels. However, therapeutic administration of OM and LAN may induce CYP1A based on findings using caffeine and theophylline as probe substrates (11–13). Furthermore, induction of CYP1A was also demonstrated in patients receiving OM by determining the microsomal cytochrome P450 profile in biopsy specimens from the liver lobules (14, 15). On the other hand, PAN has exhibited no significant induction of CYP1A2 (38), suggesting that CYP1A induction ability of PAN might be lower than those of OM and LAN in humans. Thus, the present findings indicating difference in inducibility of CYP1A between PPIs in rats may provide some usefulness for assessing enzyme induction by these drugs in humans, while their direct relevance to humans is not clear because these induction data in rats were obtained at high doses of PPIs (50 mg/kg/day and 300 mg/kg/day).

In summary, the present results suggest that enzyme induction differs among these PPIs not only quantitatively but also qualitatively, i.e. PAN induced CYP2B preferentially rather than CYP1A in contrast to OM and LAN, which induced CYP1A rather than CYP2B. Further studies are required to clarify the relationships between the liver concentration of PPIs and the induction of the P450 isozymes in human.


